Inhibition of nuclear import pathways by Mx2

Relevance: Lentiviruses such as HIV-1 are uniquely efficient in their ability to infect non-dividing cells through the hijacking of cellular nucleocytoplasmic trafficking pathways. Nuclear import of primate lentiviruses is inhibited by the interferon inducible GTPase Mx2, which localizes to the nuclear pore complex. However, the process by which HIV-1 utilizes cellular nucleocytoplasmic trafficking and how nuclear entry is inhibited by Mx2 remain poorly defined. This proposal seeks to determine the nuclear import pathways that are inhibited by Mx2, how its antiviral activity is affected by heterogeneity in cellular nucleocytoplasmic trafficking, and how it localizes to the nuclear pore complex. These studies will provide fundamental insights into the precise mechanism by which an innate immune effector inhibits a crucial step in HIV-1 infection.

Background

Cellular nucleocytoplasmic trafficking - Transit of large molecules between the cytosol and the nucleus is controlled by nuclear pores, which are large multi-protein complexes composed of thirty distinct nucleoporins organized in repetitively arranged subcomplexes with 8-fold rotational symmetry. The NPC has a tripartite architecture, with a central major architectural scaffold, that includes the transmembrane Nups, the Nup107, Nup93, and Nup62 subcomplexes. The NPC is appended with cytoplasmic filaments, and the nuclear basket [Fig. 1 and (1)]. Although often considered as static structures, NPCs are actually quite dynamic, with varied composition across the nuclear envelope (2), and altered expression under certain conditions, such as upon interferon (IFN) stimulation (3).

Transport of substrates through the NPC is regulated by members of a family of nuclear transport receptors (NTRs) known as karyopherins (and/or importins/transportins). NTRs bind cargoes via the recognition of a nuclear localization signal (NLS) or nuclear export signal (NES) to form a transport complex, which is (generally) regulated by the GTPase, Ran. There is spectacular diversity in NLS/NES sequences, often making the precise identification of these sequences and their cognate importin challenging. Furthermore, there is also a large variety in the mechanisms regulating nuclear transport, including, but not limited to, intra/intermolecular masking of NLS/NES, changes in transport factor expression, and changes in NPC composition (4).

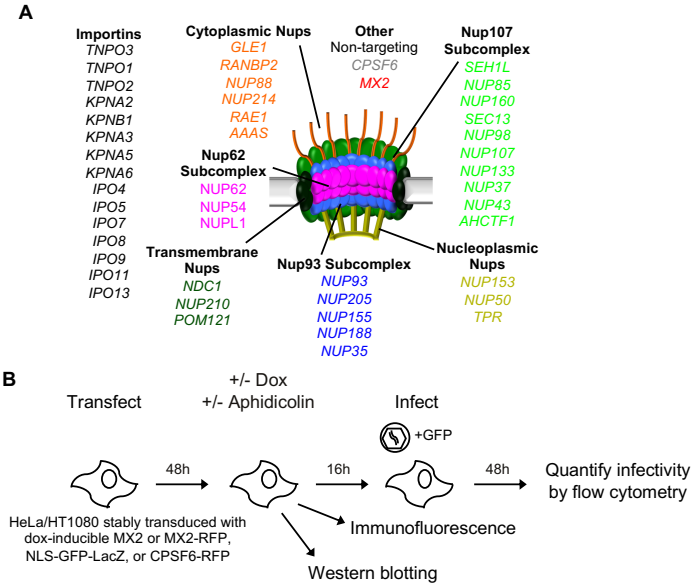

Figure 1. (A) Schematic representation of the nuclear pore complex and genes included in siRNA library color coded by subcomplex. Importins/nuclear transport receptors (NTRs) included in the siRNA library are listed in black. Also included, siRNA targeting MX2 or CPSF6 and a non-targeting control siRNA. (B) Experimental strategy to investigate the roles of Nups and NTRs involved in HIV-1 infection, NLS function, Mx2 subcellular localization, and anti-viral activity of Mx2.

Nuclear entry of HIV-1 - Access to the chromosomal DNA contained within the nucleus of target cells is critical for retroviral integration and replication. Among retroviruses, the lentiviruses are uniquely efficient in their ability to enter the nucleus of interphase cells, in which the nuclear membrane is intact. The viral capsid (CA) is the key viral determinant of the ability of HIV-1 to infect non-dividing cells (5). In addition to CA itself, cellular CA-interacting proteins have been suggested to regulate or otherwise influence HIV-1 nuclear import and infection, including the peptidylprolyl isomerase cyclophilin A (CypA) and the mRNA processing protein cleavage and polyadenylation specificity factor 6 (CPSF6) (6). CA can also directly interact with multiple Nups (6-8) and CA-dependent interactions with Nups appear to play a role in determining where HIV-1 proviral DNA is integrated into the host genome, perhaps by directing HIV-1 preintegration complexes to specific nuclear import pathways or through effects on chromatin architecture (6).

The antiviral protein Mx2 - Mx2 is an IFN-induced GTPase that localizes to the NPC (9-12) and inhibits HIV-1 infection by preventing nuclear import of the viral preintegration complex. Mx2 contains an NLS-like sequence in its first 25 amino acids that is required for NPC localization and the inhibition of HIV-1 infection (10, 11, 13). Dimerization, but not higher order oligomerization is required for the antiviral activity of Mx2 (14, 15), and transfer

of the N-terminal domain that contains both the specificity determinants and the NLS to heterologous proteins that form multimers (Mx1, Fv1, and Arfaptin2) but do not normally inhibit HIV-1 infection, confers anti-HIV-1 activity [(7, 16) and Table 1]. The viral CA is the major determinant of Mx2 sensitivity, and CA mutations that confer partial or complete Mx2 resistance are also known to affect interactions with cellular proteins implicated in HIV-1 nuclear import (6, 8). These mutants also alter infection in non-dividing cells and/or integration site selection, suggesting the possibility that they may utilize distinct pathways to access nuclear DNA.

Recent work has indicated that Mx2 can also inhibit nuclear import of herpesviruses (17, 18). Importantly, while GTP binding and hydrolysis by Mx2 are dispensable for anti-HIV-1 activity, they are required for herpesvirus restriction (17, 18). On the other hand, the N-terminal domain is required for restriction in both cases. This suggests that while Mx2 can inhibit nuclear import of highly divergent viruses, distinct mechanisms underlie this inhibition.

Approach

Preliminary Results:

Nuclear pore heterogeneity affects HIV-1 infection and Mx2 activity – Shortly after the initial identification of Mx2 as an antiviral protein, we determined that there are significant cell-type and cell-cycle-dependent differences in the sensitivity of both wild-type and CA-mutant viruses to Mx2's antiviral activity (7). We then established that there is striking variability in Nup expression among cell lines commonly utilized in HIV-1 investigation (and in primary T cells and macrophages) (7) that has the potential to affect HIV-1 infection and its modulation by cell-cycle and Mx2. In order to determine the importance of Nups and NTRs on HIV-1 infection and the antiviral activity of Mx2 in variable cell contexts, we used a panel of siRNAs targeting human Nups and NTRs (Fig. 1A). HeLa or HT1080 cells stably transduced with doxycycline-inducible Mx2 were transfected with siRNAs before splitting into replicate wells for Mx2 induction and growth arrest followed by western blotting to confirm knockdown, immunofluorescence to determine Mx2 localization, or infection with wild-type or CA-mutant HIV-1 reporter virus to measure effects on HIV-1 infection and the antiviral activity of Mx2 [Fig. 1B and (7)]. The results of these experiments that relevant for this proposal are summarized in Figure 2. Briefly, Nup and NTR depletion affected HIV-1 infection and both the subcellular localization and antiviral activity of Mx2. Importantly, the effects of these depletions were also CA sequence, cell-type, and cell-cycle dependent, indicating complex and varied functional interactions between the HIV-1 CA, Nups, and Mx2. Overall, our findings are consistent with a model in which several Nup-dependent 'pathways' to HIV-1 nuclear entry and integration exist.

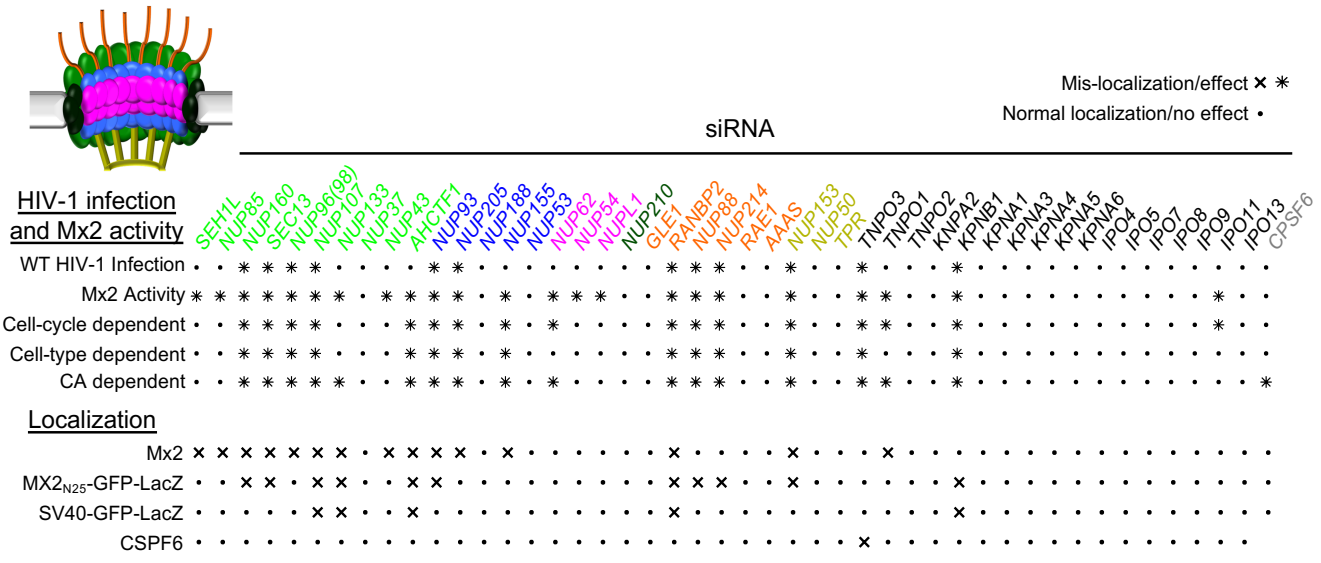

Figure 2. Summary of the effects of Nup/NTR depletion. Top: Nup/NTR depletions with effects on HIV-1 infection and Mx2 activity as well as depletions in which the effects were cell-cycle, cell-type, or HIV-1 CA dependent. Bottom: Summary of the localization of Mx2-RFP, CPSF6-RFP, or NLS-GFP-LacZ fusions upon siRNA transfection. (Mx2 and CSPF6 localization was monitored in HeLa and HT1080 cells, NLS-fusions were tested in HeLa cells only). Adapted from (7)

Different NLS sequences have distinct Nup requirements - To assess nuclear pore function under conditions of Nup knockdown, we generated cell lines expressing GFP-LacZ fusion proteins appended with one of two different NLS sequences (since GFP-LacZ alone is excluded from the nucleus), the canonical SV40 large T-

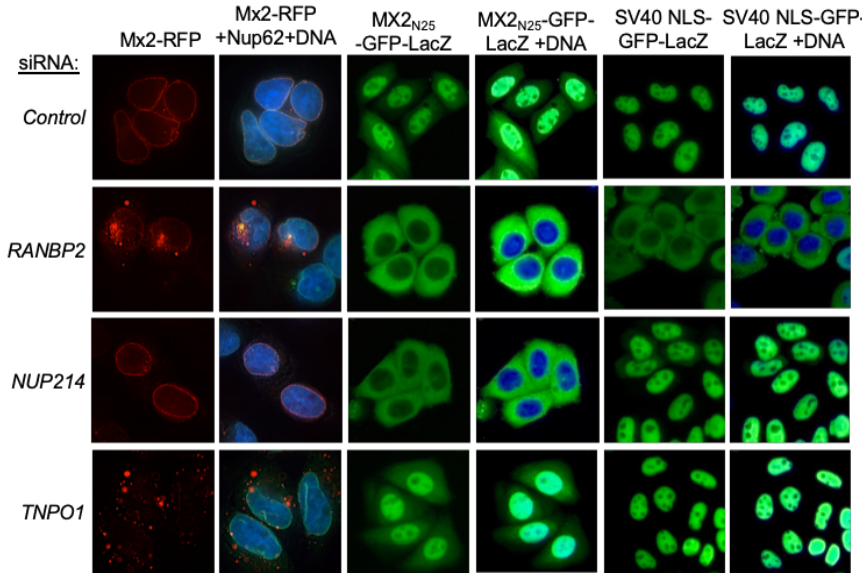

Figure 3. Localization of Mx2-RFP, $Mx2_{N25}$GFP-LacZ, and SV40NLS-GFP-LacZ in HeLa cells transfected with the indicated siRNA. Adapted from (7).

antigen NLS or the N-terminal 25 amino acids of Mx2, both of which localized GFP-LacZ to the interior of the nucleus [Fig. 3 and (7)]. Depletion of Nups and NTRs revealed that most perturbations allowed residual nuclear transport to proceed. Indeed, the nuclear accumulation of the SV40 NLS-GFP-LacZ was unaffected or only marginally affected by most Nup or NTR depletions while a larger number of Nup depletions cause partial or apparently complete inhibition of $Mx2_{N25}$-GFP-LacZ nuclear accumulation (Figs. 2 and 3). Additionally, nuclear localization of CSPF6 was maintained following all Nup/NTR depletions, with the except of TNPO3, [Fig. 2 and (7)] known to be required for nuclear localization of CPSF6 (6).

Nup/NTR requirements for Mx2 activity and localization are context-dependent – As shown in Fig. 3, the nuclear interior localization of $Mx2_{N25}$-GFP-LacZ contrasted with the nuclear pore localization of Mx2 itself, and with the nuclear pore localization of $Mx2_{NTD}$-Mx1 [Table 1 and (16)], Additionally, while transfer of the N-terminal domain of Mx2 to the dimeric cellular protein Arfaptin2 conferred anti-viral activity, $Mx2_{N91}$-Arfaptin2 appears to localize at the trans Golgi network [Table 1 and (7)] similar to authentic Arfaptin2 (19). This suggests that some property of Mx2 and Mx1 confers nuclear pore entrapment on a signal that would direct transport of an otherwise inert cargo to the nuclear interior. Furthermore, Mx2 and $Mx2_{N25}$-GFP-LacZ exhibited differential Nup/NTR requirements for their subcellular localization [Figs. 2, 3 and (7)]. For example, while depletion of TNPO1 abrogated the NPC localization and antiviral activity of Mx2, it had no effect on the subcellular localization of $Mx2_{N25}$-GFP-LacZ (Figs. 2 and 3), or on the antiviral activity of $Mx2_{N91}$-Arfaptin2 [Table 1 and (7)].

Table 1. Summary of Mx2 fusion proteins relevant to this investigation.

Fusion or Mutant	Localization	Anti-HIV-1 activity	TNPO1-dependent	References
Mx2	NPC	Yes	Yes	(7, 9-12)
$Mx2_{\Delta N25}$	Cytoplasm	No	-	(9,10)
$Mx2_{N25}$-GFP-LacZ	Nucleus	No	No	(7)
$Mx2_{N25}$-Mx1	NPC	Yes	?	unpublished
$Mx2_{N91}$-Mx1	NPC	Yes	?	(16)
$Mx2_{N25}$-ARFIP2	trans Golgi network	Yes	-	unpublished
$Mx2_{N91}$-ARFIP2	trans Golgi network	Yes	No	(7)
$Mx2_{N91}$-$ARFIP2_{K226E/Q227E}$	?	?	?	-

Mx2 can inhibit nuclear transport of non-viral cargos in an import pathway dependent manner – We next tested whether Mx2 could disrupt specific cellular nuclear import pathways as defined by different NLS sequences. To this end, we attached various NLS signals to a GFP-LacZ fusion protein and monitored whether Mx2 expression affected the nuclear accumulation of each NLS-GFP-LacZ variant [Fig. 4 and (7)]. The varied Mx2 sensitivity of different NLS sequences indicates that Mx2 does not induce a global block to nuclear import but can inhibit the movement of a non-viral cargo protein from the cytoplasm to the nucleus in a manner that is highly dependent on the nature of the NLS, and by inference, the 'pathway' taken into the nucleus.

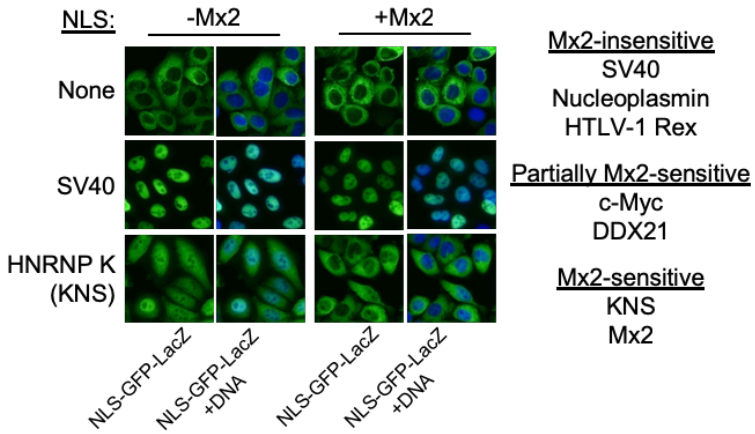

Figure 4. Left: Localization of NLS-GFP-LacZ fusions in HeLa cells in the presence or absence of Mx2. Right: Summary of sensitivity of NLS function to Mx2 activity. Adapted from (7).

AIM 1. Define the requirements for the inhibition of nuclear import by Mx2.

Rationale – Our preliminary work indicates that Mx2 preferentially inhibits specific nuclear import pathways, in particular those favored by HIV-1. However, neither the features of the import pathways that are inhibited by Mx2 nor the role of NPC heterogeneity in this inhibition have been defined. In this aim, we will investigate the determinants for Mx2-mediated blocks to nuclear import.

Aim 1.1 What are the determinants for Mx2's ability to inhibit various nuclear import pathways? – Our initial panel of NLS-GFP-LacZ fusions revealed that Mx2 expression prevents the nuclear accumulation of a subset of these NLSs [Figure 4 and (7)]. In order to better understand the pathways inhibited by Mx2, we will first test the sensitivity of an additional set of NLS sequences fused to GFP-LacZ with distinct transport factor requirements or sub-nuclear targeting (Table 2). These fusions have already been generated in our laboratory and are awaiting testing.

Table 2. Additional nuclear localization signals that will be fused to GFP-LacZ and monitored for their subcellular localization under multiple conditions by immunofluorescence

NLS (Parent Protein)	Rationale
M9 core (hnRNP A1)	TNPO1-dependent
SR (CPSF6)	TNPO3-dependent
NPCT (Nup153)	Targets to NPC
M9-like (Nup153)	Nup153-TNPO1 interaction
NLS2 (cGAS)	KPNA2-dependent
aa201-246 (Lamin B receptor)	Targets to inner nuclear membrane

The recent finding that Mx2 inhibits herpesvirus nuclear import, and that this inhibition requires GTPase activity raise the possibility that there are distinct determinants in Mx2 required to inhibit specific nuclear import pathways. To test this hypothesis, we will test the ability of Mx2 mutants to inhibit the nuclear import of Mx2-sensitive cargo. Specifically, we express Mx2 mutants with that affect NPC targeting [ΔN25, RRR-$AAA_{11\text{-}13}$ (10, 20)], GTP binding and hydrolysis [K131A, T151A (11)], and higher-order oligomerization [YRGK-$AAAA_{487\text{-}490}$ (Table 3 and (14))] and monitor their ability to inhibit our panel of NLS-GFP-LacZ fusions. We will also test the whether $Mx2_{NTD}$- fusion proteins ($Mx2_{N91}$-Mx1 and $Mx2_{N91}$-Arfaptin2) have similar effects on nuclear import of non-viral cargo.

Aim 1.2 What are the Nups and transport factors required for import of Mx2-sensitive NLSs? – Our preliminary data demonstrated that Nup/NTR depletion has specific effects on NLS function. Therefore, we will utilize our custom siRNA library to determine the Nup/NTR requirements for nuclear targeting of Mx2 sensitive and insensitive NLSs. We will also monitor the ability of Mx2 to inhibit NLS function upon Nup/NTR depletion. Although these siRNA-mediated depletions cannot ascribe a specific function to an individual Nup due to pleiotropic effects, the comprehensive nature of this approach does provide a framework for comparing nuclear import pathways. Importantly, comparison of NLS function to HIV-1 infection upon Nup depletion will also provide insights into how HIV-1 hijacks cellular nucleocytoplasmic trafficking for access to the chromatin and the mechanism of Mx2's inhibition of this process.

Aim 1.3 How do cell-type and cell-cycle affect Mx2's ability to inhibit various nuclear import pathways? – Our recent investigation revealed that HIV-1 nuclear import is more complex than previously appreciated and that the role of specific Nups in viral infection and Mx2 activity are affected by cell-type and cell-cycle (7). However, the preliminary experimentation on NLS function and Mx2 sensitivity has only been conducted in dividing HeLa cells. As such, we will also perform the above experiments in HT1080 cells (which have notable difference in the effects of cell cycle, CypA, CA mutations, Mx2, and Nup expression on HIV-1 infection), and in growth-arrested HeLa and HT1080 cells. To facilitate the generation and unbiased analysis of this data, images will be collected using a fluorescence microscope with a programmable motorized stage and advanced imaging software in the laboratory of my mentor, Dr. Carolyn Coyne.

We first identified Mx2 as an IFN-inducible factor that inhibits HIV-1 infection in the monocytic THP-1 cell line. These cells can be differentiated into an adherent macrophage like-state by treating with phorbol 12-myristate 13-acetate (PMA) that accentuates the effect of type I IFN on HIV-1 infection (10). We can therefore utilize these cells to test the effect of Mx2 expression on nuclear import in macrophage-like cells by transducing THP-1 cells containing with doxycycline-inducible Mx2 with our NLS-reporter constructs followed by PMA stimulation and induction of Mx2 expression. We will also utilize these cells to investigate the ability of endogenous Mx2 to inhibit various nuclear import pathways by examining nuclear import in IFN-treated cells expressing a control or Mx2-targeting shRNAs (10). Importantly, these experiments will also determine whether there are Mx2-independent effects of IFN treatment on nuclear import due to changes in NPC composition (3).

AIM 2. Identify the determinants that localize Mx2 to the NPC.
Rationale – Our preliminary work indicates that although the N-terminal domain of Mx2 is required for localization to the NPC and can transfer antiviral activity to heterologous proteins, it is not sufficient for NPC localization. Furthermore, distinct Nups and transport factors are required for the localization and anti-viral activity of full-length Mx2 and $Mx2_{NTD}$- fusion proteins. In this aim, we will examine the requirements for Mx2's subcellular localization and interaction with cellular transport factors.

Aim 2.1 How does oligomerization affect the subcellular localization of Mx2? – Based on our observation (and reports from other groups) that while Mx2 and $Mx2_{N25}$-Mx1 [or $Mx2_{N91}$-Mx1] localize to the NPC, both $Mx2_{N25}$-GFP-LacZ and monomeric Mx2 localize to the nuclear interior [Fig. 3, Table 1, and (7, 15, 16)], we hypothesize that oligomerization is required for NPC targeting of Mx2. In order to test this hypothesis, we will examine the subcellular localization of Mx2 and $Mx2_{N91}$-Mx1 proteins (with C-terminal RFP or other tags) containing mutations that prevent dimerization or higher-order oligomerization (Table 3). Additionally, since the trans Golgi targeting signal of Arfaptin2 in our $Mx2_{N91}$-ARFIP2 fusion appears to override the NLS sequence (at least to our detection abilities), we will generate fusions in which Golgi targeting is abolished but structural integrity is maintained [$Mx2_{N91}$-$ARFIP2_{K226E/Q227E}$ (19)] to determine whether these dimeric fusions localize to the NPC.

It is possible that oligomerization is not the determining factor in localization of Mx2 to the NPC. If oligomerization mutants and other mutants described here (such as GTPase mutants) also exhibit NPC targeting, we will investigate the subcellular localization of additional Mx2 mutants (eg. ΔL4 loop) and generate more Mx2-Arfaptin2 and/or Fv1 fusion constructs containing larger portions of Mx2 sequence in order to identify the requirements for NPC targeting.

Table 3. Summary of oligomerization mutants for this study

	Monomer	**Dimer**	**References**
Mx2	M574D or Y651D	$YRGK\text{-}AAAA_{487\text{-}490}$	(14,15)
$Mx2_{N91}$-Mx1	M527D	R408D or G392D	(14)

Aim 2.2 What determines the requirement for TNPO1-dependence of Mx2 localization and antiviral activity? – In agreement with work from the laboratory of Dr. Michael Malim (20), our recent report indicated that TNPO1 is required for NPC targeting of Mx2 and for the inhibition of HIV-1 infection in a cell-cycle and CA-dependent manner [Fig. 2 and (7)]. However, inhibition of HIV-1 infection by the trans Golgi localized $Mx2_{N91}$-ARFIP2 fusion and nuclear targeting of $Mx2_{N25}$-GFP-LacZ are TNPO1-independent [Table 1 and (7)]. Utilizing the Mx2 mutants and $Mx2_{NTD}$- fusion proteins previously described in this proposal, we will investigate the requirements for TNPO1-mediated NPC targeting and antiviral activity. First, we will determine if TNPO1 depletion (or CRISPR knockout) affects NPC targeting of $Mx2_{N91}$-Mx1 or Mx2 mutants identified in the above experiments to localize to the NPC or nuclear interior. Next, we will determine if TNPO1 depletion has an effect on the inhibitory activity of our antiviral Mx2 mutants or fusions by testing their ability to inhibit HIV-1 infection in TNPO1-depleted cells. Finally, by comparing these results, we will assess whether there is a functional link between the role of TNPO1 in NPC localization and antiviral activity of Mx2, or whether these interactions can be uncoupled.

Additional ongoing work related to this proposal – While siRNA mediated knockdown has provided significant insight concerning the importance of specific Nups in HIV-1 infection, such depletions have significant pleiotropic effects and often dramatically alter the formation/structure of the NPC (7), thereby confounding the interpretation of results. Since the domains and structures of many Nups have been well defined, this allows for the precise construction of deletion mutants and for the modification of the functional capabilities of the nuclear pore without disrupting nuclear pore assembly. As part of a multi-investigator project led by Dr. Tom Hope at Northwestern University for which funding is pending, we are currently employing CRISPR-based approaches to truncate endogenous Nups in HeLa and HT1080 cells and determine the effects of these manipulations on HIV-1 infection. These Nup-mutant cell lines will also be useful tools for defining specific nuclear import pathways and assessing the requirements for Mx2 activity. As such, although there is no overlap in the experimentation proposed in each of these investigations, they are highly complementary and together will foster my scientific and professional development as an independent investigator in the HIV field.

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